MicroRNA-223 controls the expression of histone deacetylase 2: a novel axis in COPD

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Abstract: Reduced activity of histone deacetylase 2 (HDAC2) has been described in patients with chronic obstructive pulmonary disease (COPD), but the mechanisms resulting in decreased expression of this important epigenetic modifier remain unknown. Here, we employed several in vitro experiments to address the role of microRNAs (miRNAs) on the regulation of HDAC2 in endothelial cells. Manipulation of miRNA levels in human pulmonary artery endothelial cells (HPAEC) was achieved by using electroporation with anti-miRNAs and miRNA mimics. Target prediction software identified miR-223 as a potential repressor of HDAC2. In subsequent stimulation experiments using inflammatory cytokines known to be increased in patients with COPD, miR-223 was found to be significantly induced. Functional analysis demonstrated that overexpression of miR-223 decreased HDAC2 expression and activity in HPAEC. Conversely, HDAC2 expression and activity was preserved in anti-miR-223-treated cells. Direct miRNA-target interaction was confirmed by reporter gene assay. In a next step, reduced expression of HDAC2 was found to increase the levels of the chemokine fractalkine (CX3CL1). In vivo studies confirmed elevated expression levels of miR-223 in mice exposed to cigarette smoke and in emphysematous lung tissue from LPS-treated mice. Moreover, a significant inverse correlation of miR-223 and HDAC2 expression was found in two independent cohorts of COPD patients. These data emphasize that miR-223, the most prevalent miRNA in COPD, controls expression and activity of HDAC2 in pulmonary cells, which, in turn, might alter the expression profile of chemokines. This pathway provides a novel pathogenic link between dysregulated miRNA expression and epigenetic activity in COPD. KEY MESSAGES: Histone deacetylase 2 is directly targeted by miR-223. Levels of miR-223 are induced by interleukin-1 and tumor necrosis factor- . miR-223 controls the expression of fractalkine by targeting histone deacetylase 2. miR-223 levels are increased in COPD mouse models. miR-223 levels inversely correlate with HDAC2 expression in COPD patients.

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microRNA-223 controls the expression of histone deacetylase 2: a novel axis in COPD

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Abstract

Reduced activity of Histone Deacetylase 2 (HDAC2) has been described in patients with chronic obstructive pulmonary disease (COPD) but the mechanisms resulting in decreased expression of this important epigenetic modifier remain unknown. Here we employed several *in vitro* experiments to address the role of microRNAs (miRNAs) on the regulation of HDAC2 in endothelial cells. Manipulation of miRNA levels in human pulmonary artery endothelial cells (HPAEC) was achieved by using electroporation with anti-miRNAs and miRNA mimics. Target prediction software identified miR-223 as a potential repressor of HDAC2. In subsequent stimulation experiments using inflammatory cytokines known to be increased in patients with COPD miR-223 was found to be significantly induced. Functional analysis demonstrated that overexpression of miR-223 decreased HDAC2 expression and activity in HPAEC. Conversely, HDAC2 expression and activity was preserved in anti-miR-223 treated cells. Direct miRNA-target interaction was confirmed by reporter gene assay. In a next step, reduced expression of HDAC2 was found to increase the levels of the chemokine Fractalkine (CX_3CL1). *In vivo* studies confirmed elevated expression levels of miR-223 in mice exposed to cigarette smoke and in emphysematous lung tissue from LPS treated mice. Moreover, a significant inverse correlation of miR-223 and HDAC2 expression was found in two independent cohorts of COPD patients. These data emphasize that miR-223, the most prevalent miRNA in COPD, controls expression and activity of HDAC2 in pulmonary cells, which, in turn, might alter the expression profile of chemokines. This pathway provides a novel pathogenic link between dysregulated miRNA expression and epigenetic activity in COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by irreversible airflow limitation, chronic inflammation and emphysematous destruction of the lung tissue. The social and economic burden of COPD is substantial: COPD affects about 10% of the world's population and, according to prediction models, will become the third leading cause of death by 2020 [1]. The pathogenesis of COPD, however, is incompletely understood. Cigarette smoke and other noxious particles are important risk factors by inducing the release of inflammatory mediators such as tumor necrosis factor (TNF) α [2], interleukin 1 (IL-1) β [3] and chemokines [4]. These factors, among other mechanisms, lead to infiltration of the airway epithelium with inflammatory cells, chronic bronchial inflammation and consecutive tissue destruction [5].

The expression pattern of major transcription factors downstream of inflammatory cytokines appears to be epigenetically changed. Modifications of histone proteins are – together with DNA methylation – the most important group of epigenetic alterations [6]. Of these, the balance between histone acetylation and deacetylation is arguably the most investigated. Decreased histone deacetylase (HDAC) activity allows the acetylated chromatin to be unbound to histones. In turn, transcription factors can access the chromatin and the mRNA of distinct inflammatory cytokines and chemokines can be produced [7].

In lung tissue obtained from patients with COPD, the activity of HDAC and especially HDAC2 has been found decreased. Moreover, HDAC2 activity was discovered to be negatively correlated with disease severity [8]. This is an interesting observation with major impact on the pathogenic understanding and novel therapeutic approaches to patients with COPD. To date, however, it remains unclear which mechanisms are responsible for the reduced expression of HDAC2 in COPD.

Of interest, HDAC2 has previously been described to be influenced by micoRNAs (miRNAs) in liver cancer [9]. miRNAs are endogenously expressed small non-coding RNA molecules of 20-22nt that bind to complementary sequences within the 3' untranslated region (UTR) of target mRNAs in order to repress their expression [10]. It is assumed that more than 60% of the human genome is regulated by miRNAs [11]. Conversely, the role of miRNAs in the pathogenesis of COPD is only poorly understood and the potential impact of miRNAs on the expression of HDAC2, a key player in the pathogenesis of COPD [8], and on the inflammatory response in human pulmonary cells remains unknown.

The present study was designed to test the hypothesis that HDAC2 is regulated via miRNA and the potential of these miRNAs to maintain a chronic inflammatory response in COPD.

Material and Methods

Human patient samples

Lung tissue samples from COPD patients were obtained through our tissue bank in collaboration with the Division of Thoracic Surgery at the University Hospital of Zurich. In the present study patients with severe COPD that fulfilled the Global Initiative for COPD (GOLD) criteria were included. Written informed consent was obtained from all patients. The study design was approved by Zurich's ethical review committee (KEK ZH-Nr 2014-0011). Validation of human gene expression was performed in patient samples derived from the GLUCOLD study cohort measured by Affymetrix Human Gene ST array and Affymetrix miRNA 2.0 array (GSE36221) [12-14].

Cell culture

Human hepatocellular carcinoma (HepG2) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% HEPES. The HepG2 cell line was provided by the Department of Clinical Pharmacology and Toxicology of the University Hospital Zurich [15]. Human pulmonary artery endothelial cells (HPAEC, from Gibco, Life Technologies, Zug, Switzerland), human pulmonary artery smooth muscle cells (HPASMC, from Gibco), normal human bronchial epithelial cells (NHBEC from Lonza, Basel, Switzerland), and monocytes (Mono-Mac-6 cells from DSMZ, Braunschweig, Germany) were cultured according to the manufacturer's instructions. All cells were incubated at 37°C in 5% CO_2 atmosphere. When reaching 80% confluence, adherent cells were trypsinized in trypsin-EDTA and prepared for experiments. In all experiments performed, cells from passage 5-9 were used. Cells were seeded and allowed to settle for at least 24 hours (h) before being transfected or stimulated. Cultured cells were stimulated either with IL-1β, TNF-α or a cocktail of both with a concentration of 10ng/ml, respectively. All stimulation agents are purchased from Sigma Aldrich (Buchs, Switzerland). Stimulation was performed over different time points (24h, 48h, or 72h).

Experimental in vivo models

To induce lung emphysema and COPD in an animal model, mice were exposed to cigarette smoke from research cigarettes (1R4F, University of Kentucky, USA) for 5 days per week as described elsewhere [16]. After 3 weeks mice were euthanized and total RNA including miRNAs from lung tissue was isolated. In the second model, mice were treated either with lipopolysaccharide (LPS) or control phosphate buffer saline (PBS) inhalants as described before to induce elastase which leads to the development of lung emphysema [17]. Paraffin embedded mouse lung tissue samples were used for quantification of miRNAs. Both animal models were provided by our collaborators Benjamin Marsland and Giovanni Camici. The animal experimentation was approved by the local Canton's Veterinary office.

Molecular biology methods

A detailed description of several molecular biology methods including plasmid construction, reporter gene assay, transfection, RNA isolation from tissue, cultured cells, supernatants, and paraffin blocks, quantification of gene expression and mature miRNAs, Western Blot, and HDAC/HDAC2 Glo assay can be found in the electronic supplementary material of this article.

Data processing

All data are presented as the mean ± standard deviation (SD) of the indicated number of observations. Parametric or non-parametric distribution of data was determined using the Kolmogorov–Smirnov test. The paired or unpaired t-test (two-tailed) as a parametric method for testing was applied. Correlation analysis of normal distributed data was carried out using Pearson's one-tailed correlation test reflecting the inverse relation of the expression of miRNAs and their associated targets. A p-value <0.05 was considered to be statistically significant (*≤0.05, **≤0.01, ***≤0.001). Statistical evaluations were performed with the software package GraphPad Prism Version 5.0a (GraphPad Software, La Jolla, CA, USA).

Results

miR-223 directly targets HDAC2

To address the question whether the observed reduction of HDAC2 protein in COPD patients could be mediated by the action of miRNAs, DICER1, a key protein in miRNA processing, was silenced in HPAEC using siRNA directed against DICER1. As shown in Figure 1, levels of DICER1 mRNA were significantly decreased after 24h (reduction of 72%), 48h (reduction of 55.3%), and 72h (reduction of 46.3%). Simultaneously, a trend toward increased levels of HDAC2 mRNA was observed after 24h (increase of 46.3%), 48h (increase of 21.2.%), and 72h (increase of 13.4%) of transfection. Moreover, when the activity of HDAC2 was assessed in HPAEC transfected for 24h with siRNA targeting DICER1 increased HDAC2 activity was found (increase to 1.44±0.53, p=0.101, Figure 1C). These data indicate a negative correlation between the expression levels of HDAC2 and DICER1 implying a potential miRNA mediated mechanism of HDAC2 regulation in HPAEC.

To determine which miRNA could be involved in HDAC2 repression, the following strategy was applied: (i) search for HDAC2 targeting miRNAs using computational algorithms [18], and (ii) focus on miRNAs previously associated with COPD. Based on this strategy three miRNAs were selected for further experiments: miR-182, miR-223 and, as a positive control, miR-145 that has been recently shown to target HDAC2 [9].

Next, a luciferase reporter gene assay was used to investigate whether selected miRNAs directly interact with HDAC2. Reporter gene assay was carried out in HepG2 cells since these cells show high transfection efficacy. In addition, the interaction of miR-145 with HDAC2 was reported in HepG2 cells [9]. After transfection of luciferase constructs comprising the entire 3'UTR of HDAC2 and co-transfection of mimics of miR-145, miR-182, and miR-223 luciferase activity was measured. As presented in Figure 2A, miR-145 significantly decreased luciferase activity of the construct encoding part 2 of HDAC2 (reduction to 0.77±0.18, p=0.046) whereas miR-223 significantly repressed the activity of the construct encoding part 2 (reduction to 0.7±0.11, p=0.004) and part 3 (reduction to 0.76±0.14, p=0.042). In contrast, miR-182 failed to decrease luciferase activity of any of the used constructs. These data indicate that the 3'UTR of HDAC2 contains functional seed matches of miR-223.

Inflammatory cytokines induce the levels of miR-223

To further investigate the role of miR-223 in COPD, the levels of miR-223 were quantified in pulmonary cells. As shown in Figure 3A, miR-223 levels could be measured and detected in HPASMC, NHBEC and HPAEC. Next, stimulation experiments were performed using cytokines that have been shown to be highly upregulated in COPD: IL-1β and TNFα [2, 3]. As demonstrated in Figure 3B, stimulation of HPAEC with IL-1β and TNFα increased the levels of miR-223 at all time points assessed. A significant upregulation of miR-223 was observed when HPAEC were stimulated with a cocktail of both (48h of stimulation: 2.84±1.06 fold, p=0.018; 72h of stimulation: 3.34±1.4

fold, p=0.02) as compared with unstimulated cells. A concomitant reduction of HDAC2 expression was detected when HPAEC were stimulated with a cocktail of IL-1β and TNFα (48h of stimulation: reduction to 0.63±0.11, p=0.002; 72h of stimulation: reduction to 0.81±0.17, p=0.07, Figure 3C).

Next, the effect of cytokine stimulation on miR-223 levels was tested in other pulmonary cells and in monocytes. As shown in Figure 4A and 4B, stimulation of NHBEC and HPASMC with a cocktail of IL-1β and TNFα had no significant effect on miR-223 levels. The expression of miR-223 was significantly reduced in cell extracts of monocytes stimulated for 72h (reduction to 0.47±0.06, p<0.001, Figure 4C). Conversely, levels of miR-223 in the supernatants were found significantly increased (72h of stimulation: 1.7±0.46 fold, p=0.027, Figure 4D).

These data demonstrate an endothelial cell specific cytokine-induced effect on miR-223 expression and, moreover, indicate that after cytokine treatment monocytes might release miR-223 in the extracellular space.

miR-223 targets HDAC2 in HPAEC

In a next step, we addressed the question whether miR-223 affects HDAC2 activity in HPAEC. After transfection with mimic or inhibitor of miR-223 HDAC2 activity was measured using a total HDAC or a specific HDAC2 activity assay. Enforced expression of miR-223 resulted in a significant decrease of total HDAC (reduction to 0.73±0.15, p=0.007) and HDAC2 activity (reduction to 0.69±0.18, p=0.007) as compared to scrambled transfected control cells (Figure 5A and 5B). Conversely, miR-223 inhibition led to increased total HDAC and HDAC2 activity, however, without reaching statistical significance (Figure 5A and 5B).

Regarding the expression of HDAC2, the same samples were analyzed for mRNA and protein levels of HDAC2. Transfection of HPAEC with miR-223 decreased the mRNA levels of HDAC2 to 0.86±0.15 (p=0.099) whereas miR-223 inhibition significantly increased mRNA levels of HDAC2 by 1.08±0.036 fold (p=0.007) as compared to control cells (Figure 5C and 5D). When protein levels of HDAC2 were analyzed, a significant decrease of HDAC2 protein levels in miR-223 mimic transfected HPAEC was observed (reduction of 31±19%; p=0.021, Figure 5E). However, treatment with miR-223 inhibitor did not significantly influence the protein levels of HDAC2 (Figure 5E).

miR-223 induces the expression of Fractalkine via HDAC2

To investigate the downstream effect of decreased HDAC2 activity, the mRNA levels of Fractalkine (CX_3CL1), a chemokine expressed in endothelium, was assessed. As presented in Figure 6, a tendency to an increased expression of CX_3CL1 in HPAEC transfected with miR-223 mimic was observed (Figure 6A). Similarly, treatment with anti-miR-223 led to a reduction of the mRNA level of CX_3CL1 (Figure 6B).

The role of miR-223 *in vivo*

To confirm a dysregulated expression of miR-223 and its target HDAC2 *in vivo*, levels of miR-223 were measured in two experimental models of lung emphysema and COPD. First, mice were treated with LPS to induce lung inflammation and lung dysfunction [17] and the levels of miR-223 in lung tissue were found significantly upregulated as compared to saline-treated control mice (fold change of 1.97±0.12, p=0.006, Figure 7A). These data were confirmed in a small number of cigarette smoke exposed mice showing increased levels of miR-223 (fold change of 1.63±0.62, n=3, Figure 7B). Moreover, human lung tissue from COPD patients was analyzed for miR-223 and HDAC2 expression. As presented in Figure 7C, a negative correlation between the expression of HDAC2 and the levels of miR-223 was found in patients with severe COPD (r=-0.532, p=0.046, n=11) collected

in our own tissue bank at the University Hospital Zurich. Similar results were obtained by analyzing patient samples from the databank of the GLUCOLD study [12] confirming a negative correlation between miR-223 and its target HDAC2 (r=-0.224, p=0.047, n=57, Figure 7D).

Discussion

In this study we provide evidence of a novel role for miR-223 in the pathogenesis of COPD. In detail, we show that (i) HDAC2 is directly targeted by miR-223 through binding to seed matches located in the 3'UTR of its mRNA transcript, (ii) total HDAC and HDAC2 activity is repressed in pulmonary endothelial cells in response to miR-223 overexpression, (iii) inflammatory cytokines known to be upregulated in COPD patients significantly induce the levels of miR-223 in endothelial cells, (iv) miR-223 is upregulated in experimental models of the disease, and that (v) the levels of miR-223 negatively correlate with the expression of HDAC2 in human lung tissue of COPD patients. These data further indicate that exposure to inflammation increases the levels of miR-223 in the lung endothelium, which, through repression of HDAC2 and release of chemokines, might provide a positive inflammatory feedback loop in the pathogenesis of COPD. Taken together, our findings offer a novel mechanistic explanation for the previously described downregulation of HDAC2 in patients with COPD [8].

The expression and activity of HDAC2 was previously shown to be repressed in COPD patients and, as such, was found to be linked to disease severity [8]. While previous studies reported that nitration of tyrosine residues affect activity and stability of HDAC2 protein [19] and, moreover, that proteosomal imbalance might be of critical importance for these processes [20], the regulation of HDAC2 gene expression and associated pathways in COPD have not completely been unraveled. miRNAs have emerged as post-transcriptional gene silencers and we thus suggested their involvement in the decreased expression of HDAC2. In the context of COPD, miR-223 was recently described as the most upregulated miRNA in lung tissue of patients but its function has not been addressed [21]. Several miRNA target prediction algorithms identified potential binding sites of miRNAs that included seed matches for miR-223 in the 3'UTR of HDAC2 [18]. Moreover, the interaction of miRNAs with HDAC2 has previously been described in liver cancer where miR-145 was reported to act as tumor suppressor by targeting HDAC2 [9]. Due to these facts, the direct interaction of different miRNAs expressed in pulmonary cells and predicted to bind to the 3'UTR of HDAC2 was investigated. Subsequent reporter gene analysis confirmed the interaction of miR-145 and, as a novel finding, of miR-223 with the 3'UTR of HDAC2. We further identified seed matches located in the last two parts of the 3'UTR of HDAC2 indicating that these binding sites are targeted by miR-223.

These results supported our hypothesis of a post-transcriptional regulation of HDAC2 by miR-223. In functional studies, we demonstrated that enforced expression of miR-223 in pulmonary endothelial cells leads to a significant reduction of protein levels and activity of HDAC2 in these cells. The mRNA levels, however, remained almost unchanged suggesting a miRNA-mediated inhibition of the translational process of the targeted transcript. Such a mechanistic interplay is frequently observed in the context of miRNA mediated gene regulation in human disease including pulmonary disorders [22]. Of note, when miR-223 was silenced using miRNA-inhibitors only minor effects could be observed on the expression levels of HDAC2. This finding might be explained as a consequence

of the low basal expression levels of miR-223 in unstimulated pulmonary endothelial cells derived from healthy donors, in which no additional suppression can be achieved by the antagonization of miR-223.

Chronic inflammation in COPD is orchestrated by the activity of cytokines and chemokines, of which many can be detected at increased levels in the circulation of patients [5, 23]. In stimulation experiments using endothelial cells that, *in vivo*, reflect the first responders of the lungs to these proinflammatory signals, we found that IL-1β and TNFα significantly induced the levels of miR-223 in a time-dependent manner. These cytokines have been described as key inflammatory mediators in COPD [2, 3] and their activity in COPD patients might explain the significant upregulation of miR-223 levels *in vivo* as shown by Ezzie and colleagues [21]. Both cytokines are activators of the transcription factor nuclear factor kappa B (NF-κB). This signaling pathway has previously been proposed as link between cigarette-smoke induced inflammation, cytokine release and histone acetylation [24]. Our data support this notion (Figure 8) but at this moment it remains unclear whether the cytokine-triggered induction of miR-223 is a downstream event of the NF-κB signaling or, alternatively, acts as an independent factor.

As a consequence of our stimulation experiments showing that miR-223 is most inducible in endothelial cells and the fact that these cells might act as pulmonary gatekeeper for inflammatory cells and cytokines and, as such, provide the interface between lung tissue and blood circulation the focus of this study was set on HPAEC. Endothelial cells secrete chemoattracants (e.g. CX_3CL1) and might control inflammation within the lung tissue. The interaction between monocytes and endothelial cells is further highlighted by the fact that, upon stimulation with inflammatory cytokines, miR-223 is released from monocytes. Such mechanism might indicate exosomal transfer of miR-223 to endothelial cells as suggested previously [25] and, potentially, to other lung cells. Moreover, HPAEC are of further interest since endothelial dysfunction in smokers is well recognized [26, 27] and might contribute to altered inflammatory signaling. Since no effect on the expression levels of miR-223 has been observed in stimulation experiments using smooth muscle and bronchial epithelial cells, a cell-type specific effect of miR-223 induction upon cytokine stimulation cannot be excluded. As such, it remains elusive at the moment whether these mechanisms apply also to alveolar macrophages, in which reduced HDAC2 activity has initially been described [8].

As functional readout downstream of reduced HDAC2 activity, we observed an increase of CX_3CL1 (Fractalkine) in HDAC2-deficient endothelial cells. Fractalkine is an important chemokine with a well-established role in the context of COPD and cigarette-smoke induced inflammation [4, 28]. As membrane-anchored protein, Fractalkine acts as an adhesion molecule on the surface of the endothelium, while its soluble form attracts inflammatory cells along a chemical gradient [29]. Here we identified a regulatory pathway in pulmonary endothelial cells involving inflammatory cytokines, miRNAs and HDAC2 that result in increased expression of Fractalkine (Figure 8). Our data show that stimulation of HPAEC with IL-1β and TNFα increases the expression levels of miR-223. The association between chronic inflammation and increased miR-223 levels was further emphasized in experimental models of COPD in which mice either treated with LPS or exposed to cigarette smoke showed elevated levels of miR-223 in their lungs when compared to control mice. Increased levels of miR-223 were further found to reduce both expression and activity of HDAC2 *in vitro*. Of note, a negative correlation of miR-223 levels with HDAC2 expression was found in lung specimens of COPD patients from two independent tissue banks. According to Ito

and colleagues, less HDAC2 activity contributes to inflammatory processes in lung disease [8]. These results were confirmed here by showing the upregulation of Fractalkine following transfection of miR-223 mimics. This is a novel observation and highlights the importance of epigenetic modulation in the progression of COPD, which, as indicated by these experiments, might be mediated by the action of miRNAs [30].

Conclusion

To the best of our knowledge, this is the first report that has characterized the underlying mechanisms of noncoding RNAs involved in decreased HDAC2 activity in COPD patients. We provide evidence that one of the most prevalent miRNA in lung tissue (i.e. miR-223) directly targets HDAC2. Reduced HDAC2 activity, in turn, induces the expression of Fractalkine. Whether miR-223 can be used as a novel drug target in the treatment of lung inflammation in the context of COPD has to be addressed by further studies.

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Conflict of Interest

None declared

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Figure Legends

Fig. 1 Increased expression of histone deacetylase 2 (HDAC2) in DICER1-depleted endothelial cells
Human pulmonary artery endothelial cells (HPAEC) transfected with small interfering RNA directed against the miRNA-processing enzyme DICER1 showed higher expression levels of HDAC2 as compared to scrambled control transfection. mRNA expression of DICER1 a) and HDAC2 b) after 24h, 48h, and 72h of transfection was quantified using qPCR (n=6). c) HDAC 2 activity after 24h of transfection was found to be significantly increased as assessed by enzymatic luminescent assay (n=5). Statistical analysis by paired Student's t-test (*$p<0.05$, ***$p<0.001$).

Fig. 2 The 3' UTR of HDAC2 is directly targeted by miRNAs
The entire 3'UTR of HDAC2 was amplified out of genomic DNA, fragmented in three overlapping parts of approximately 2000bp length and subsequently cloned into a dual luciferase-expressing vector. a) Reporter gene studies showed that transfection of miR-223 mimic significantly lowered the relative luciferase activity of the constructs encoding part 2 and 3 as compared to scrambled (scr) control transfection. Enforced expression of miR-145 resulted in significantly repressed luciferase expression of construct encoding part 2. *Firefly* and *renilla* luciferase activity was measured using dual luciferase assay (n=4). b) The predicted binding sites of miR-223 within the 3'UTR of HDAC2 are presented. Statistical analysis by paired Student's t-test (*$p<0.05$, **$p<0.01$).

Fig. 3 Basal and cytokine-induced expression levels of miR-223 in pulmonary cells
a) Expression levels of miR-223 in HPAEC, human pulmonary artery smooth muscle cells (HPASMC), and normal human bronchial epithelial cells (NHBEC) are shown (n=4). b) Stimulation of HPAEC with inflammatory cytokines interleukin-1β (IL-1β), tumor necrosis factor α (TNFα) or a cocktail of both further increased the expression levels of miR-223 (n=5). c) Stimulation of HPAEC with the cocktail resulted in a significant decrease of HDAC2 mRNA expression after 24h and 48h (n=5). The levels of miR-223 and HDAC2 were quantified using qPCR. Statistical analysis by paired Student's t-test (*$p<0.05$, **$p<0.01$).

Fig. 4 Cytokine-induced expression levels of miR-223 in pulmonary cells and monocytes
Levels of miR-223 were assessed by qPCR in NHBEC (a, n=5) and in HPASMC (b, n=3) after stimulation with an inflammatory cytokine cocktail (IL-1β and TNFα) demonstrating no significant changes in miR-223 expression levels. Stimulation for 48h and 72h with the cytokine cocktail significantly decreased miR-223 expression levels in monocytes (c, n=5) whereas the abundance of miR-223 in the supernatants of stimulated monocytes was significantly increased after 72h of stimulation (d, n=5). The levels of miR-223 were quantified using qPCR. Statistical analysis by paired Student's t-test (*$p<0.05$. **$p<0.01$. ***$p<0.001$).

Fig. 5 miR-223 manipulation affects HDAC2 activity and expression in HPAEC

HPAEC were transfected with mimics or inhibitors of miR-223. After 72h of transfection total histone deacetylase (a) and HDAC2 (b) activity was significantly reduced in miR-223 transfected cells as compared to scrambled (scr) control transfection (n=5). Enzyme activity was measured using luminescent assays. The mRNA levels of HDAC2 were assessed in HPAEC transfected with miR-223 mimics (c) and anti-miR-223 (d). Inhibition of miR-223 yielded significantly higher mRNA expression levels of HDAC2 as compared to scrambled control transfection (n=5). d) Western Blotting experiments were used to quantify protein levels of HDAC2 in HPAEC transfected with mimics or anti-miRNA inhibitors of miR-223, respectively. After 72h of transfection enforced expression of miR-223 significantly lowered the protein amount of HDAC2 as compared to scrambled control transfection (n=5). Statistical analysis by paired Student's t-test (*$p<0.05$, **$p<0.01$).

Fig. 6 Manipulation of miR-223 alters the expression of Fractalkine

In HPAEC, a) enforced expression of miR-223 increased the mRNA levels of Fractalkine (CX_3CL1) b) whereas the inhibition of miR-223 lowered the levels of CX_3CL1. mRNA expression after 72h of transfection was measured using qPCR analysis (n=5). Statistical analysis by paired Student's t-test.

Fig. 7 miR-223 expression *in vivo*

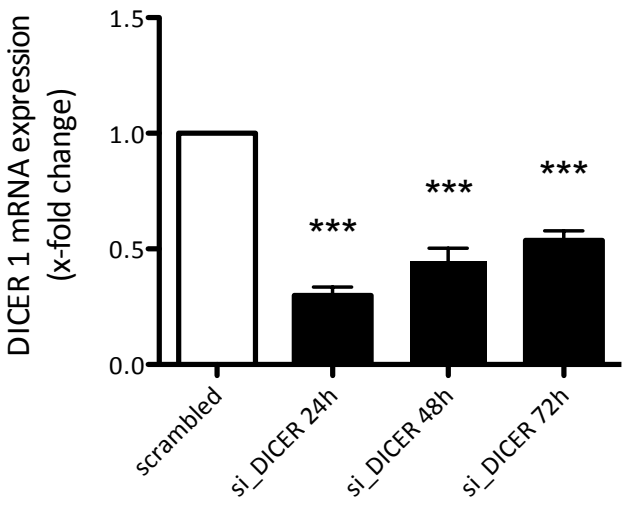
a) miRNAs were isolated from paraffin embedded lung tissue specimen of mice that have been treated with lipopolysaccharide (LPS) to induce chronic obstructive pulmonary disease (COPD). Analysis by qPCR showed that the expression levels of miR-223 were significantly upregulated in LPS treated mice as compared to saline-treated control mice (n=5). b) miRNAs were isolated from fresh total lung tissue of mice exposed to cigarette smoke. Analysis by qPCR demonstrated an increase in miR-223 levels in mice exposed to cigarette smoke compared to control mice (n=3). c) Linear regression shows a significant negative correlation between HDAC2 and miR-223 expression levels in total lung tissue from COPD patients conducted by University Hospital Zurich (n=11) These findings are confirmed in d) a COPD patient cohort from the University of Groningen (n=57). Statistical analysis by unpaired Student's t-test (**$p<0.01$) and linear regression by Pearson one-tailed correlation test in a 95% confidence interval ($p<0.05$).

Fig. 8 Proposed regulatory mechanism of miR-223 in COPD

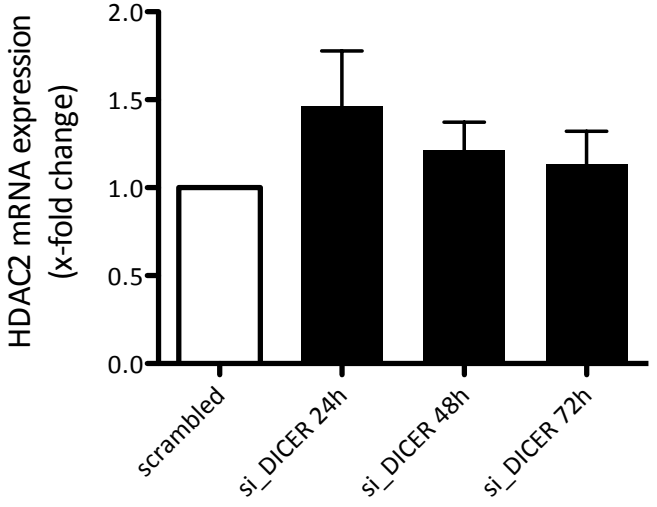
The main components of a novel regulatory pathway in pulmonary arterial endothelial cells involve the action of inflammatory cytokines, miRNAs and HDAC2. Direct silencing of HDAC2 by miR-223 result in increased expression of the chemokine Fractalkine that, in turn, might adhere more monocytes to the endothelium.

Fig. 1

a


DICER 1 mRNA expression
(x-fold change)
1.5
1.0
0.5
0.0
***
***
***
scrambled
si_DICER 24h
si_DICER 48h
si_DICER 72h

b


HDAC2 mRNA expression
(x-fold change)
2.0
1.5
1.0
0.5
0.0
scrambled
si_DICER 24h
si_DICER 48h
si_DICER 72h

c

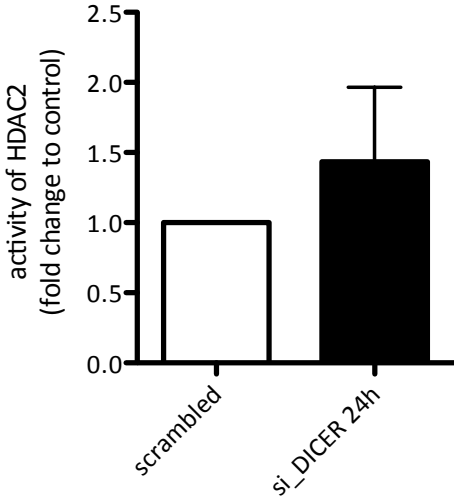
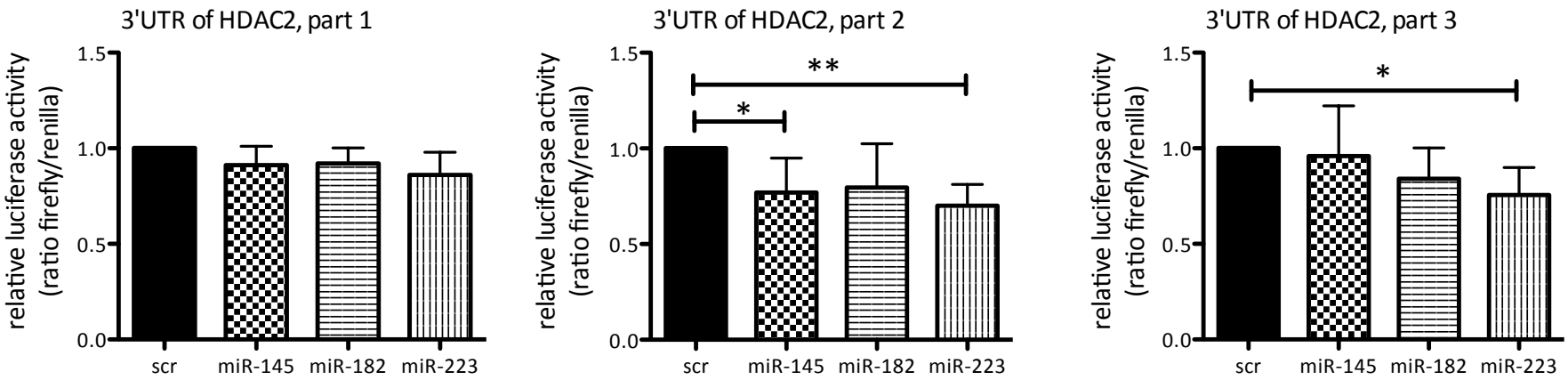

activity of HDAC2
(fold change to control)
2.5
2.0
1.5
1.0
0.5
0.0
scrambled
si_DICER 24h

Fig. 2

a



b

miR-223 binding sites:

Part 2

HDAC2: 2084 TGT GTG AAC ATA CTG AAA 2101

miR-223: 3' – ACC CCA TAA ACT– GTT TGA CTG T – 5'

HDAC2: 2428 GAT ATT TGA - - AAT TGG CA 2444

miR-223: 3' – ACC CCA TAA ACT GTT TGA CTG T – 5'

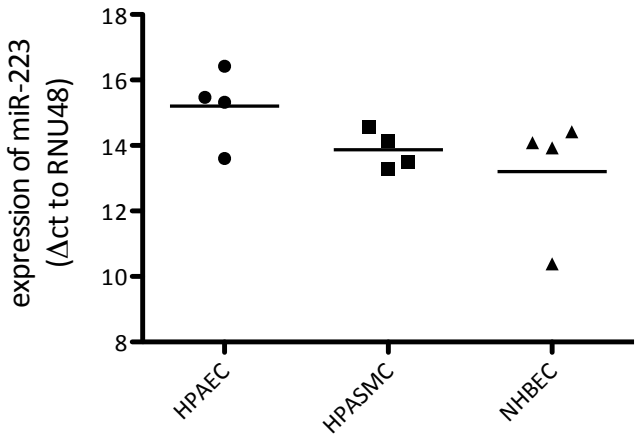
Part 3

HDAC2: 3549 TTT GGT AAA CTA AGA 3563

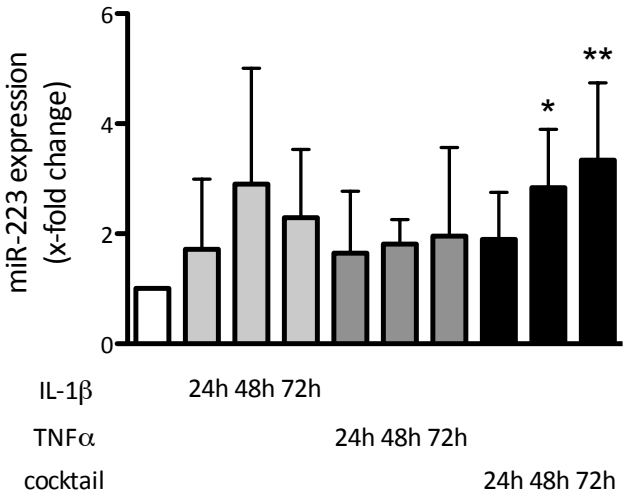
miR-223: 3' – ACC CCA TAA ACT GTT TGA CTGT – 5'

Fig. 3

a


expression of miR-223
(Δct to RNU48)
18
16
14
12
10
8
HPAEC
HPASMC
NHBEC

b


miR-223 expression
(x-fold change)
6
4
2
0
*
**
IL-1β
24h 48h 72h
TNFα
24h 48h 72h
cocktail
24h 48h 72h

c

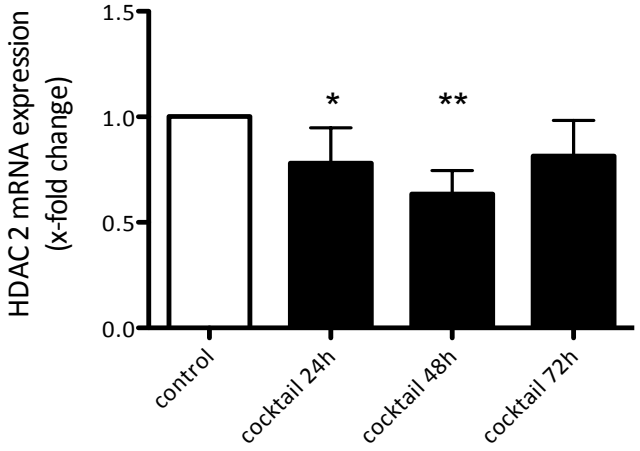
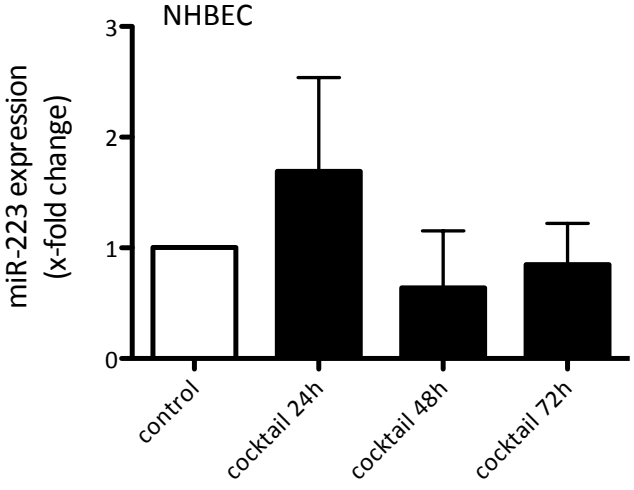
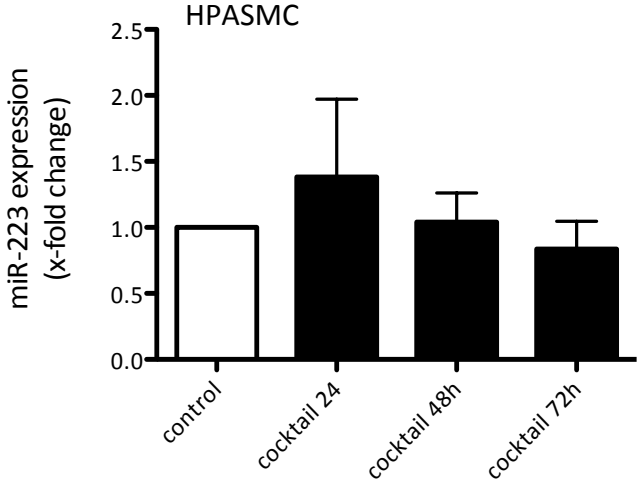

HDAC 2 mRNA expression
(x-fold change)
1.5
1.0
0.5
0.0
*
**
control
cocktail 24h
cocktail 48h
cocktail 72h

Fig. 4

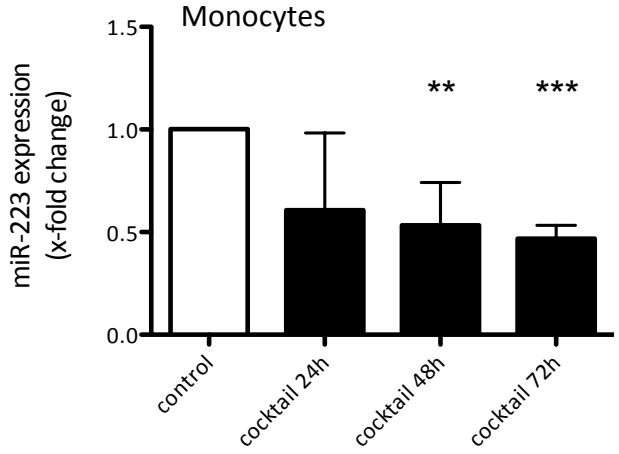
a


NHBEC
miR-223 expression
(x-fold change)
3
2
1
0
control
cocktail 24h
cocktail 48h
cocktail 72h

b


HPASMC
miR-223 expression
(x-fold change)
2.5
2.0
1.5
1.0
0.5
0.0
control
cocktail 24
cocktail 48h
cocktail 72h

c


Monocytes
miR-223 expression
(x-fold change)
1.5
1.0
0.5
0.0
**
***
control
cocktail 24h
cocktail 48h
cocktail 72h

d

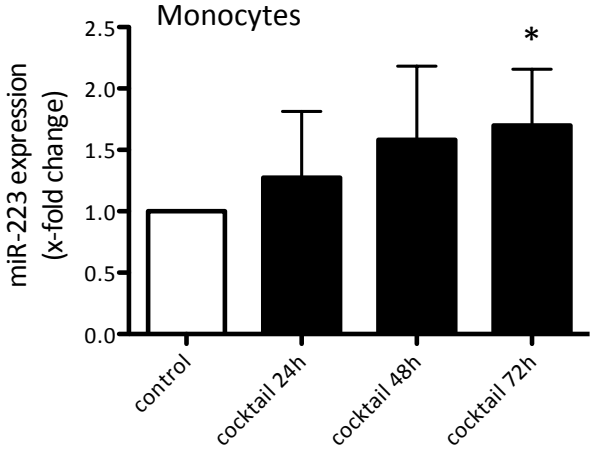

Monocytes
miR-223 expression
(x-fold change)
2.5
2.0
1.5
1.0
0.5
0.0
*
control
cocktail 24h
cocktail 48h
cocktail 72h

Fig. 5

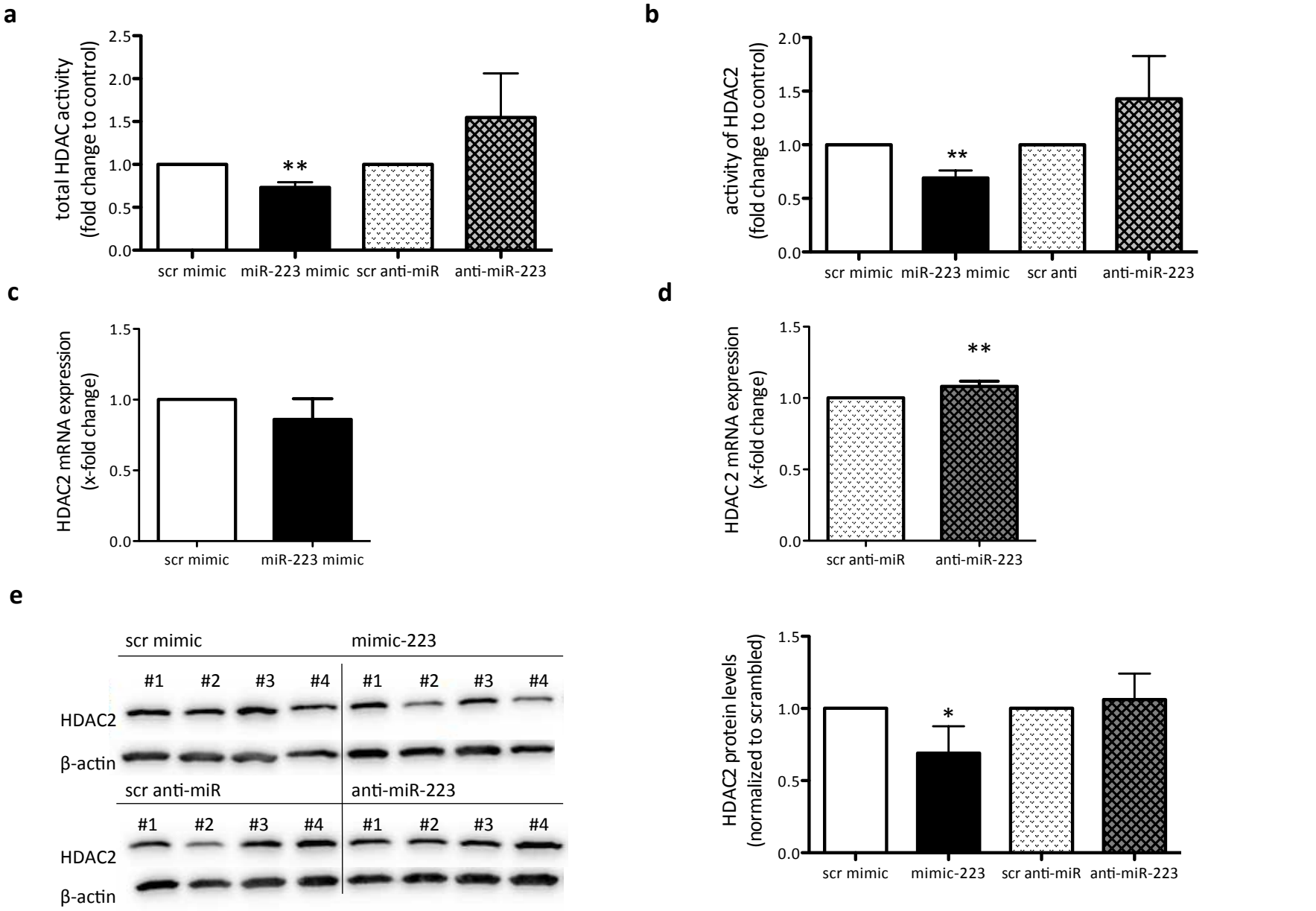
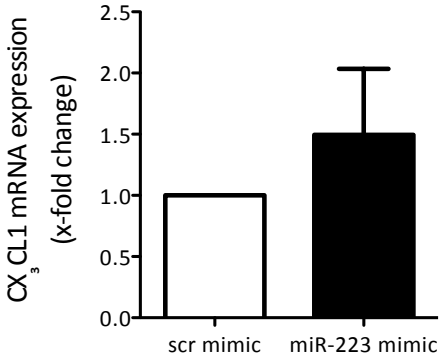

a
total HDAC activity
(fold change to control)
2.5
2.0
1.5
1.0
0.5
0.0
**
scr mimic
miR-223 mimic
scr anti-miR
anti-miR-223
b
activity of HDAC2
(fold change to control)
2.0
1.5
1.0
0.5
0.0
**
scr mimic
miR-223 mimic
scr anti
anti-miR-223
c
HDAC2 mRNA expression
(x-fold change)
1.5
1.0
0.5
0.0
scr mimic
miR-223 mimic
d
HDAC 2 mRNA expression
(x-fold change)
1.5
1.0
0.5
0.0
**
scr anti-miR
anti-miR-223
e
scr mimic
mimic-223
#1
#2
#3
#4
#1
#2
#3
#4
HDAC2
β-actin
scr anti-miR
anti-miR-223
#1
#2
#3
#4
#1
#2
#3
#4
HDAC2
β-actin
HDAC2 protein levels
(normalized to scrambled)
1.5
1.0
0.5
0.0
*
scr mimic
mimic-223
scr anti-miR
anti-miR-223

Fig. 6

a


CX3CL1 mRNA expression
(x-fold change)
2.5
2.0
1.5
1.0
0.5
0.0
scr mimic
miR-223 mimic

b

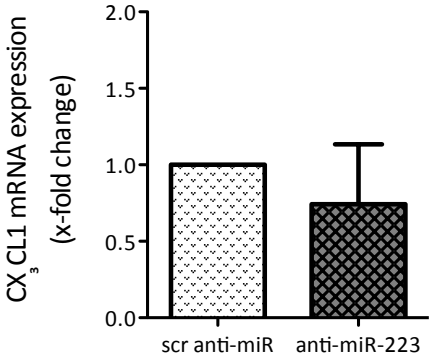

CX3CL1 mRNA expression
(x-fold change)
2.0
1.5
1.0
0.5
0.0
scr anti-miR
anti-miR-223

Fig. 7

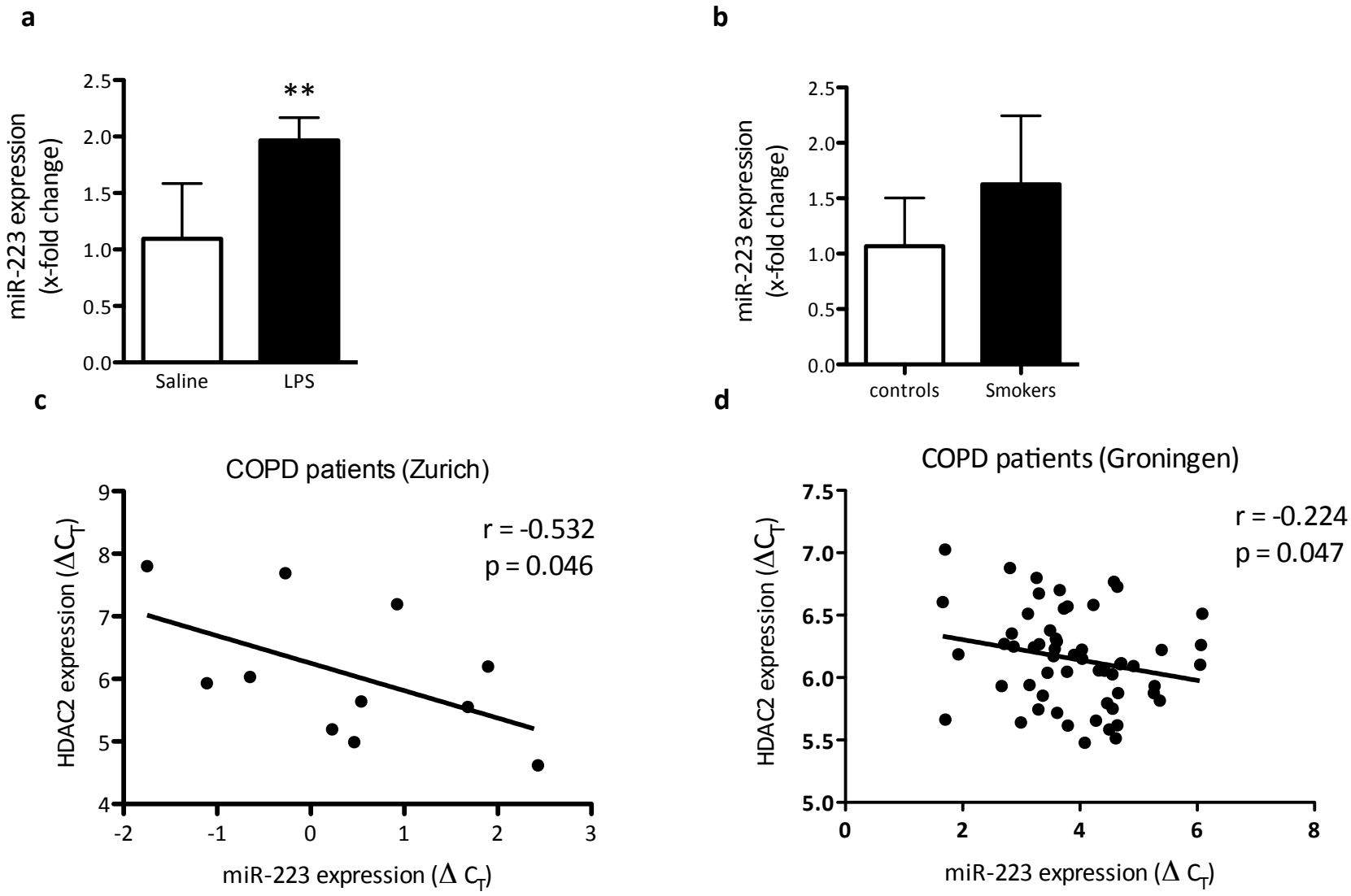
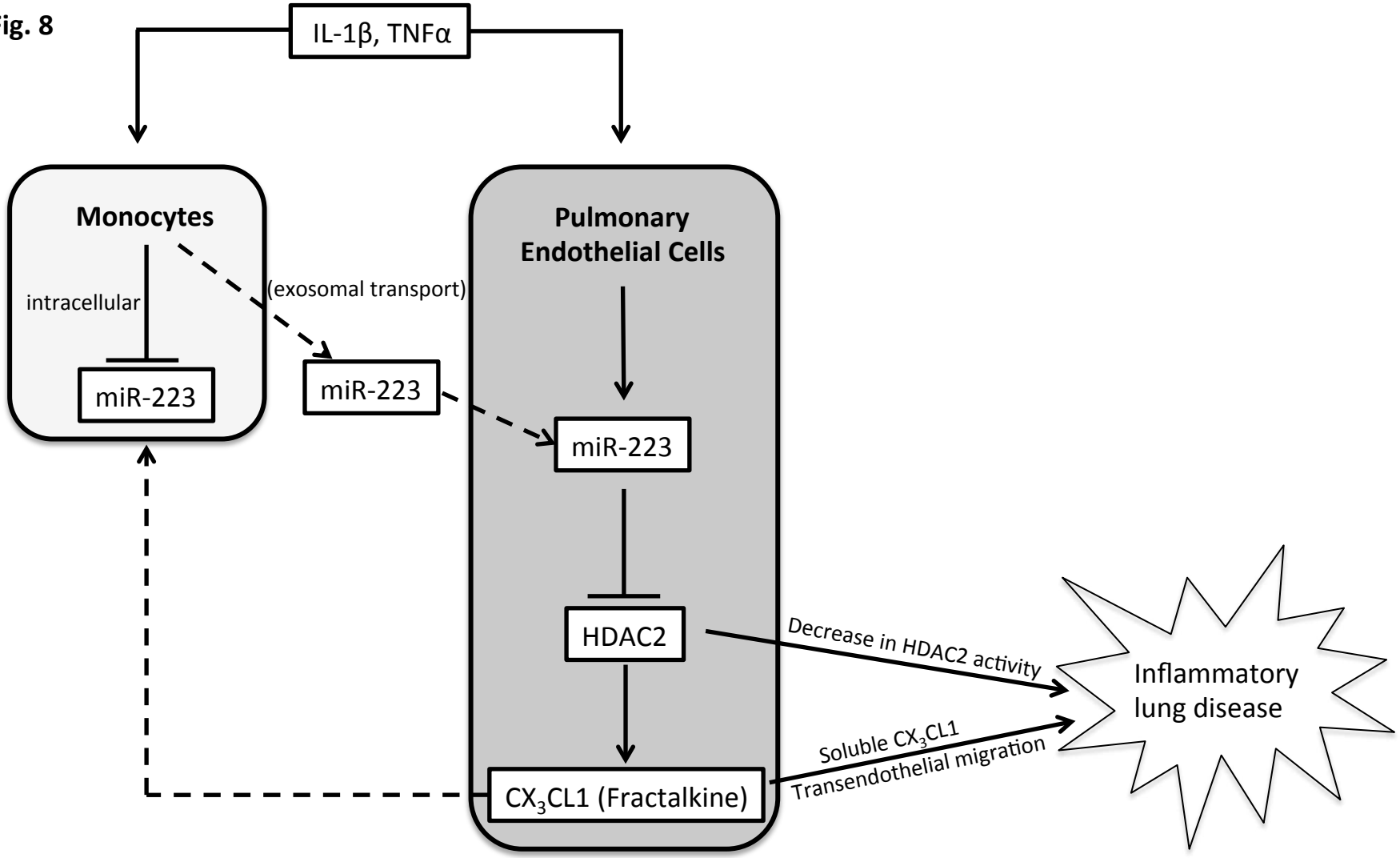

a
miR-223 expression (x-fold change)
2.5
2.0
1.5
1.0
0.5
0.0
**
Saline
LPS
b
miR-223 expression (x-fold change)
2.5
2.0
1.5
1.0
0.5
0.0
controls
Smokers
c
COPD patients (Zurich)
r = -0.532
p = 0.046
HDAC2 expression (ΔC_T)
9
8
7
6
5
4
-2
-1
0
1
2
3
miR-223 expression (Δ C_T)
d
COPD patients (Groningen)
r = -0.224
p = 0.047
HDAC2 expression (ΔC_T)
7.5
7.0
6.5
6.0
5.5
5.0
0
2
4
6
8
miR-223 expression (Δ C_T)

Fig. 8


IL-1β, TNFα
Monocytes
intracellular
miR-223
(exosomal transport)
miR-223
Pulmonary Endothelial Cells
miR-223
HDAC2
CX3CL1 (Fractalkine)
Decrease in HDAC2 activity
Soluble CX3CL1
Transendothelial migration
Inflammatory lung disease